Supplementary Materials

Supplementary materials and methods.

PB and SF neutrophil isolation: PB was obtained by venipuncture obtained by venipuncture and SF by arthrocentesis; both were collected in EDTA-containing tubes. PB was fractionated via density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). Neutrophils were isolated by dextran sedimentation of the RBC layer. SF was spun at 6,000 rpm for 10 min at 4°C. RBC lysis was performed with hypotonic salt solution. SF cell pellets and circulating neutrophils were stained with trypan blue (MP Biomedical, Solon, OH), to confirm >95% viability, then seeded on poly-L-lysine coated glass coverslips ($1x10^5$ cells/coverslip) and incubated at 37°C, 5% CO_2, for 15 min. Cells were either fixed with 4% paraformaldehyde or exposed to various conditions for 1 h, followed by fixation. Experimental conditions included incubation in the presence or absence of LPS (1ug/ml); 10% RA or healthy control sera; 10% RA or OA SF, IgG (100 µg/ml) isolated from RA/control serum or from RA/OA SF; IgM RF (100 µg/ml, see below); control human IgM (100 µg/mL; Sigma-Aldrich), recombinant human TNF-α (100 ng/mL; Pepro Tech); recombinant human IL-17 (10-100 ng/mL; eBioscience); neutralizing anti-human-TNF (5 µg/mL; Abcam); recombinant GM-CSF (10 ng/.mL; Peprotech, Rocky Hill, NJ); neutralizing anti-human-IL-17R (1µg/mL; R&D); DPI (10 µM; Tocris) (*31*), NAC (5mM; Sigma) and Cl-amidine (200 µM) (*63*).

Quantification of PB and SF NETs by fluorescent microscopy and by plate assay: NETs were quantified in neutrophil preparations from PB or SF, after staining cells with rabbit anti-human neutrophil elastase antibody (1:100) or isotype control (Abcam) for 30 min at

room temperature (RT), followed by incubation with FITC-conjugated goat anti-rabbit secondary Ab for 30 min at RT. Coverslips were mounted onto glass slides and nuclear material was stained using ProLong Gold antifade reagent with DAPI (Invitrogen). Additional Abs used to assess expression of molecules in the NETs included mouse anti-human vimentin (Thermo Scientific), mouse anti-human α-enolase (Santa Cruz), and respective IgG isotypes. Secondary Abs included rabbit Cy3 with chicken anti-goat IgG Alexa Fluor 488 or goat anti-rabbit FITC with chicken anti-mouse IgG Alexa Fluor 594 (all from Invitrogen). Slides were analyzed using an upright fluorescent microscope (BX60; Olympus). Five to ten high power images (400x) were captured using a DP70 CCD color camera (RGB, 12-bits/channel; Olympus) and DP70 controller/manager software v3.02 (Olympus). Images were loaded onto Adobe Photoshop (Adobe Systems) and two independent observers manually quantified neutrophils and NETs. NETs were identified as structures positive for both neutrophil elastase and DAPI.

To quantify NETs by plate assay, neutrophils were resuspended in RPMI without phenol red containing 0.2 μM of Sytox Green (Invitrogen). Neutrophils (1×10^4 cells) were incubated in the presence or absence of various conditions mentioned above in a 96-well dark plate for 1 h at 37°C. Fluorescence (excitation 485 nm, emission 520 nm) was measured in a Biotek Synergy H1 Hybrid Reader (Biotek) and results reported as DNA fluorescence (RFU).

Quantification of RA NETs in synovial tissue, rheumatoid nodules and skin biopsies from RA patients affected with dermatologic conditions. Five micrometer sections were

deparaffinized on a hot plate at 65°C for 1 h, and rehydrated by incubation and heat retrieval in 10mM Tris/7.5mM Boric acid/1mM EDTA/0.05% NaN_3 solution. Sections were blocked with 0.2% horse serum (Invitrogen) and stained with either rabbit anti-human neutrophil elastase or rabbit anti-human MPO (DakoCytomation). This was followed by incubation with either goat anti-rabbit FITC or chicken anti-rabbit Alexa Fluor 594 for 30 min, RT. ProLong Gold antifade reagent with DAPI was added to slides and images were captured as mentioned above. NETs were identified by two independent observers as structures positive for both DAPI and a neutrophil marker (neutrophil elastase or MPO). To quantify if IgGs contained in serum recognized Ags present in NETs, RA and control neutrophils were stimulated with LPS (1ug/mL). After fixation and overnight blocking, cells were incubated with 10% RA or control serum for 30 min at RT, followed by incubation with Texas Red-anti-human IgG (Abcam). Mounting, nuclear staining and NET quantification were performed as mentioned above. Similar experiments were performed by exposing LPS-stimulated neutrophils to citrullinated-vimentin Abs.

NET purification and quantification. Control neutrophils (1 $x10^7$) were seeded in 24-well tissue culture plates and incubated with 100 ng/mL TNF-α, 100 μg/mL RA IgG or 100 μg/mL RF in RPMI without phenol red or supplement for 1 h at 37°C. Supernatants were harvested and NETs were digested with 10 U/mL micrococcal nuclease (Thermo) for 20 min at 37°C. Supernatants were collected and spun at 300xg for 10 min to remove intact cells, followed by additional 10 min centrifugation at 16,000xg to remove debris. NET proteins were precipitated with acetone overnight at -20°C; samples were

centrifuged at 10,000xg for 30 min at 4°C, washed with 80% acetone, followed by 10 min centrifugation at 16,000xg.

Proteomics analysis of NET's content and LC/ESI MS/MS analysis, data processing and statistical analysis: Supernatants from neutrophil extracts were stored at −80°C and thawed immediately prior to proteomic analysis. An initial 5000xg centrifugation was performed at 4°C for 10 min to remove cellular debris. Soluble proteins were concentrated and buffer exchanged into 50mM ammonium bicarbonate buffer using a 3 kDa filter cut-off membrane (Amicon Ultra 3kDa MWCO, Millipore, Ireland); protein content was determined using Coomassie Protein Assay Reagent with BSA standard (Thermo Scientific). Samples were denatured in 6M urea, reduced with 5mm DTT followed by alkylation with 15mM iodoacetamide. Sequencing grade modified trypsin (Promega) at 1:20(w/w) ratio was added to samples and incubated overnight at 37°C. Tryptic digests were subjected to reverse-phase extraction prior to MS analysis.

Peptide samples were resuspended in 0.1% formic acid and loaded onto an in-house packed reverse phase separation column (0.075 × 100 mm, MAGIC C18 AQ particles, 5 µm, Michrom Bioresources). Peptides were separated on a 1% acetic acid/acetonitrile gradient system at a flow rate of 300 nL/min, and sprayed onto the MS using a nanospray source. An LTQ Orbitrap XL (Thermo Fisher) was run in automatic mode collecting high resolution MS scan (FWHM 30,000) followed by data-dependent acquisition of MS/MS scans on the 9 most intense ions (relative collision energy ~35%). Dynamic exclusion was set to collect 2 MS/MS scans on each ion and exclude it for an

additional 2 min. Charge state screening was enabled to exclude +1 and undetermined charge states. Data processing and statistical analysis are included in supplementary material.

The Human UniProt database (Release 2011-5) was appended with a reverse database and a common contaminant list. Raw files were converted to *mz*XML format and searched against the database using X!Tandem with a k-score plug-in, an open-source search engine developed by the Global Proteome Machine (http://www.thegpm.org/). Search parameters were as follows: (1) precursor mass tolerance window of 100 ppm and fragment mass tolerance of 0.8 Da; (2) allowing two missed cleavages; (3) variable modification: oxidation of methionine (+15.9949 Da) and carbamidomethyl cysteine (57.0214 Da). All proteins with a Protein and Peptide Prophet probability of greater than 0.9 were considered as positive identifications(*38*). Spectral counts were compared across the groups using one-way ANOVA and P values were adjusted for Bonferroni's multiple comparison test. Statistical analyses were performed using Graph pad prism statistical software version 5.

Cytotoxicity Assay: Lysis of neutrophils was quantified with the Cyto Tox 96® bon-radioactive cytotoxicity assay (Promega, Madison, WI), following manufacturer's protocol. In brief, supernatants of cells stimulated to undergo NET formation were incubated with the kit's substrate mix for 30 min, at room temperature. Reaction was stopped and absorbance was recorded at 490nm. Cell lysate upon exposure to Triton X was used as positive control and buffer was used as negative control. ODs were

normalized to the negative control. LDH percentage was obtained relative to positive control.

Purification of Abs to citrullinated vimentin: Recombinant vimentin (MyBioSource, LLC, San Diego, CA) was citrullinated *in vitro* with 0.2 µM PAD4 in 100mM Tris-HCl pH 7.6, 10mM $CaCl_2$, 50mM NaCl, 2mM DTT for 3 h at 37°C. Citrullinated vimentin was conjugated to activated CNBr sepharose (GE Healthcare) overnight at 4°C. Sepharose was then incubated with 1 mM ethanolamine to block unbound reactive sites. Previously purified IgG fractions from RA serum samples with high ACPA responses (>250 IU/mL; n=2 patients) were incubated with citrullinated vimentin sepharose overnight at 4°C. Bound anti-citrullinated vimentin Abs were eluted with 0.1M glycine pH 2.0 and neutralized with 1M Tris pH 7.5. Samples were dialyzed in PBS overnight at 4°C. Protein concentrations were determined using the BCA Protein Assay (Pierce).

RNA isolation and real-time quantitative PCR: RNA was extracted from 40,000 OA and RA FLS using the RNeasy kit (Qiagen). DNase was included in the RNA. RNA concentration and purity were determined by spectrophotometry. Reverse transcription was performed on 200ng of RNA from each cell type and treatment condition. cDNA synthesis was performed using a PX2 thermocycler (Thermo Hybaid) as follows: first step, one cycle of 25°C for 10 min; second step, one cycle of 37°C for 120 min; third step, one cycle extension of 85°C for 5 min.

Five µl of each cDNA product was used for each QPCR in a final reaction volume of 50 µL. Amplification cocktail consisted of TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), human GAPDH primers (Applied Biosystems, Part Number 4326317E) as the endogenous control, and sequence-specific primers (Applied Biosystems) identified by Assay ID number as follows: CCL20: Hs00171125_m1, IL-6: Hs00985639_m1, IL-8: Hs99999034_m1 and ICAM1: Hs00164932_m1. Real-time quantitative PCR was done for 45 cycles of amplification, in triplicate for each sample. Results were analyzed with Applied Biosystems 7500 software.

Supplementary figures.

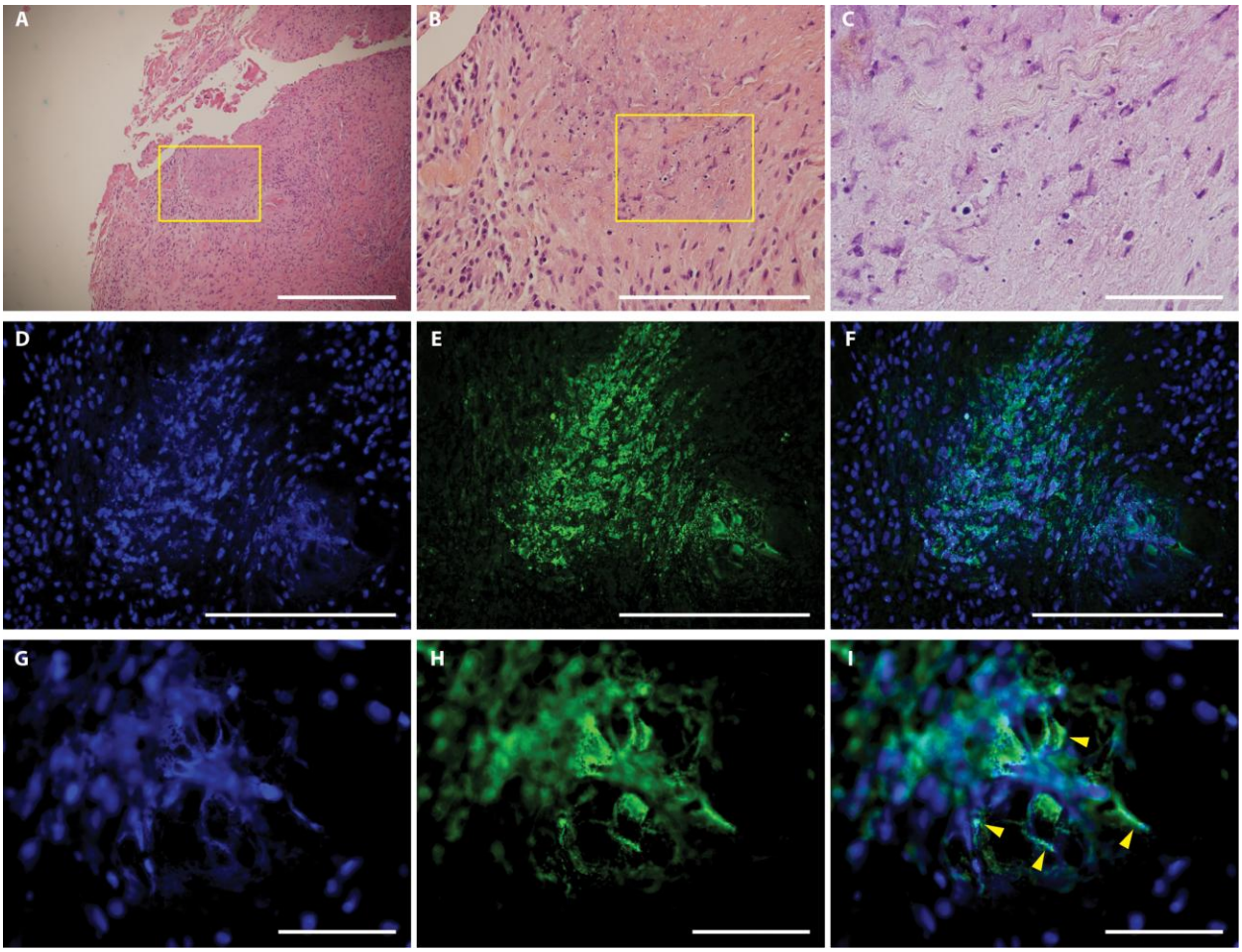
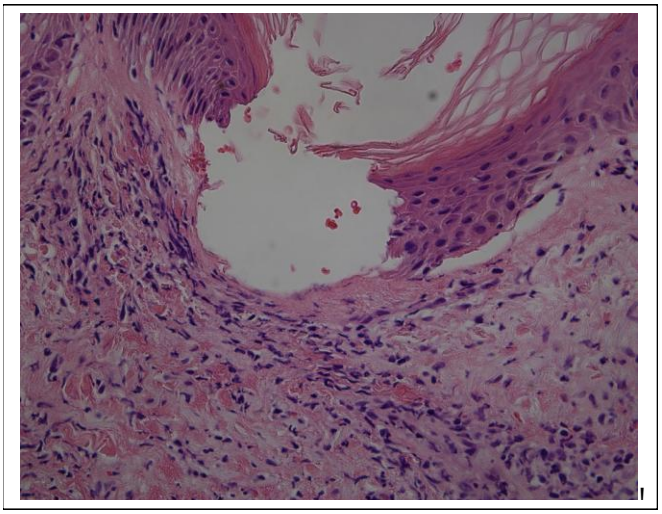


Figure S1. Netting neutrophils are identified in rheumatoid nodules. Formalin-fixed, paraffin-embedded rheumatoid nodule tissue was stained by H&E **(A-C)**; Hoechst (blue, **D** and **G**); or rabbit anti-MPO Ab followed by FITC-conjugated anti-rabbit Ab (green, **E** and **H**). Magnification is 100x (**A**, scale bar = 500 μm); 400x (**B, D-F**, scale bars = 200 μm); and 1000x (**C, G-I**, scale bars = 50 μm). Panels **F** and **I** represent overlays of **D** and **E**, and **G** and **H**, respectively. Representative areas of strand-like DNA/MPO colocalization, consistent with NETs, are demarcated by yellow arrow heads in panel **I**. Yellow boxes provide approximate orientation for subsequent magnifications.



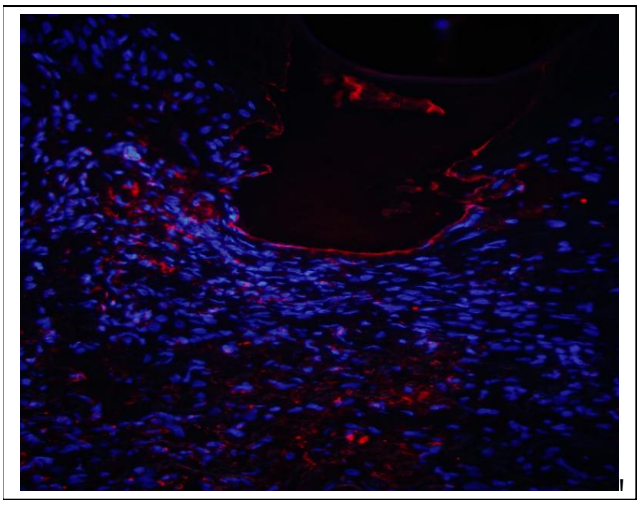
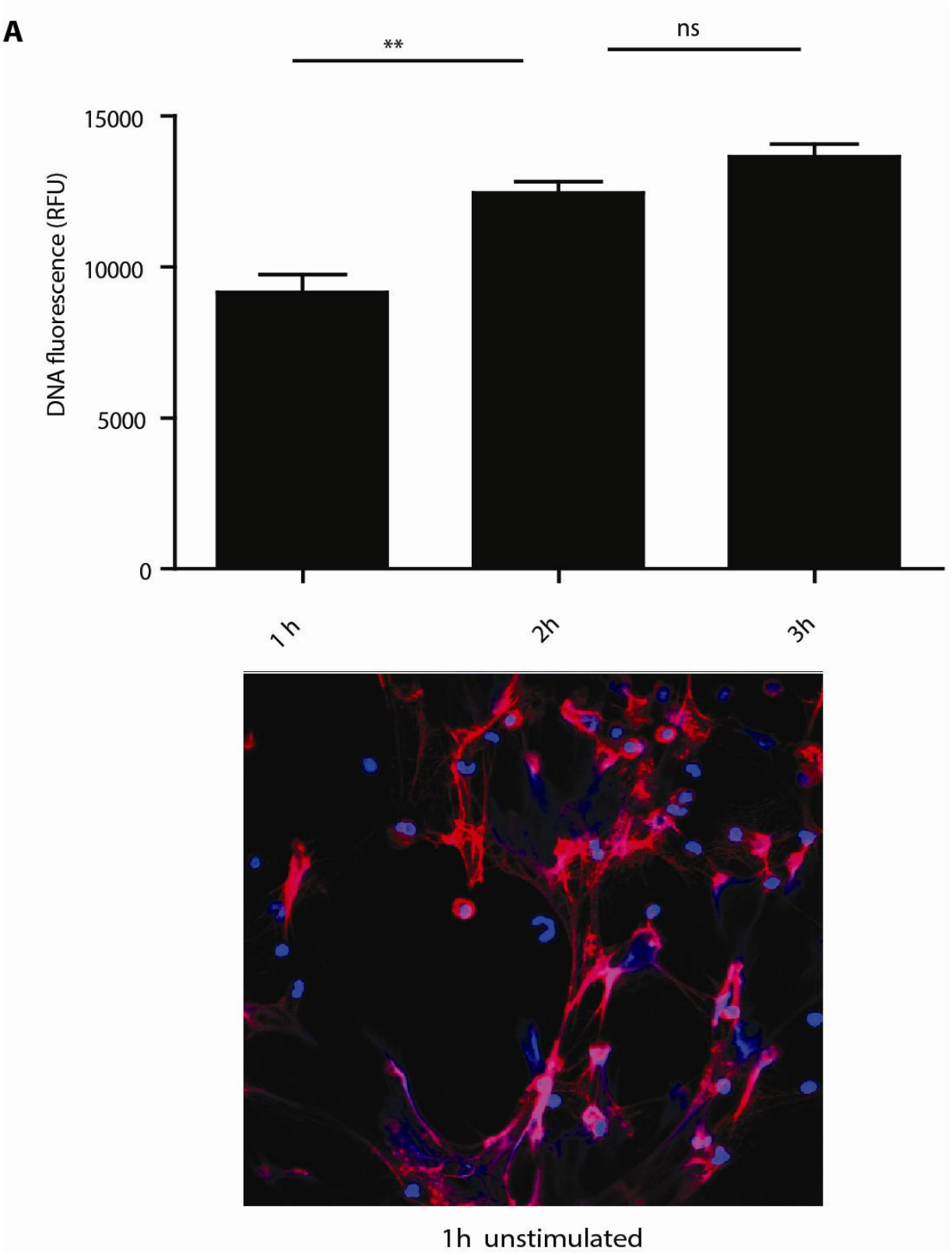


Figure S2. Netting neutrophils are present in skin from RA patients. A and **B** are sequential slices of skin from a patient with a well-characterized neutrophilic dermatosis associated with RA. A: H&E staining (400 X); B: Samples were stained with DAPI (blue) and MPO Ab (red) (400 X). Co-localization in boxed area highlights netting neutrophils.


A
**
ns
15000
10000
5000
0
DNA fluorescence (RFU)
1 h
2h
3h
1h unstimulated

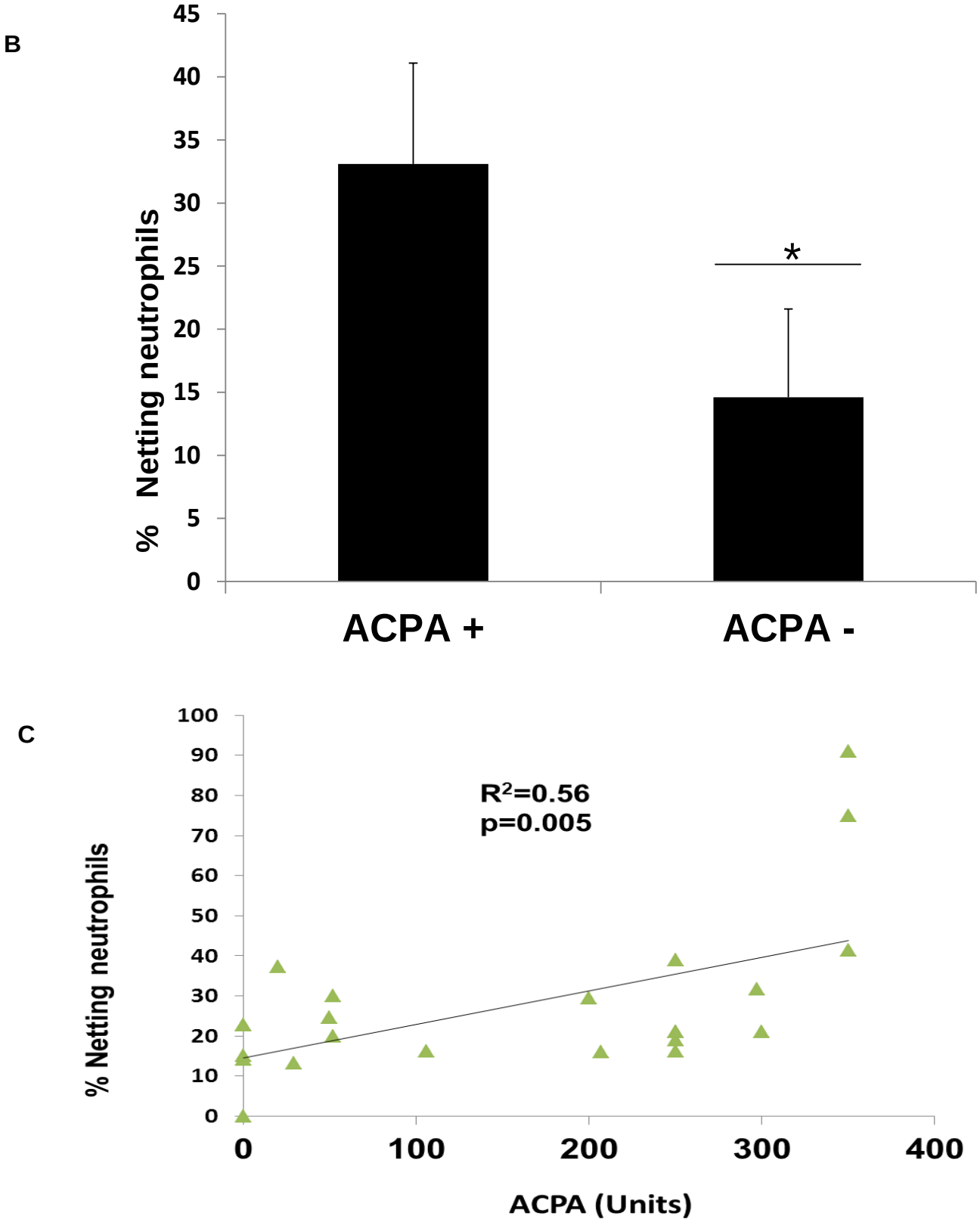


Figure S3. NET formation is accelerated in RA neutrophils and correlates with presence and titer of ACPA in RA patients. A. Significant NET formation occurs in unstimulated RA PB neutrophils within 1-3 h after isolation. Top represents mean±SEM DNA fluorescence (Sytox green assay); n=3, each performed in duplicate-triplicate; **p<0.01; ns=not significant. Bottom is a representative microphotograph (40X) displaying prominent NETosis in RA unstimulated neutrophils at 1 h in culture (red is neutrophil elastase and blue is DNA). **B.** Mean±SEM % netting unstimulated neutrophils at 1 h in culture from RA patients whose sera was ACPA+ or ACPA-. **C.** Scatterplot correlates serum ACPA levels with % NET formation in RA PB neutrophils. Pearson correlations were used to examine associations between continuous variables

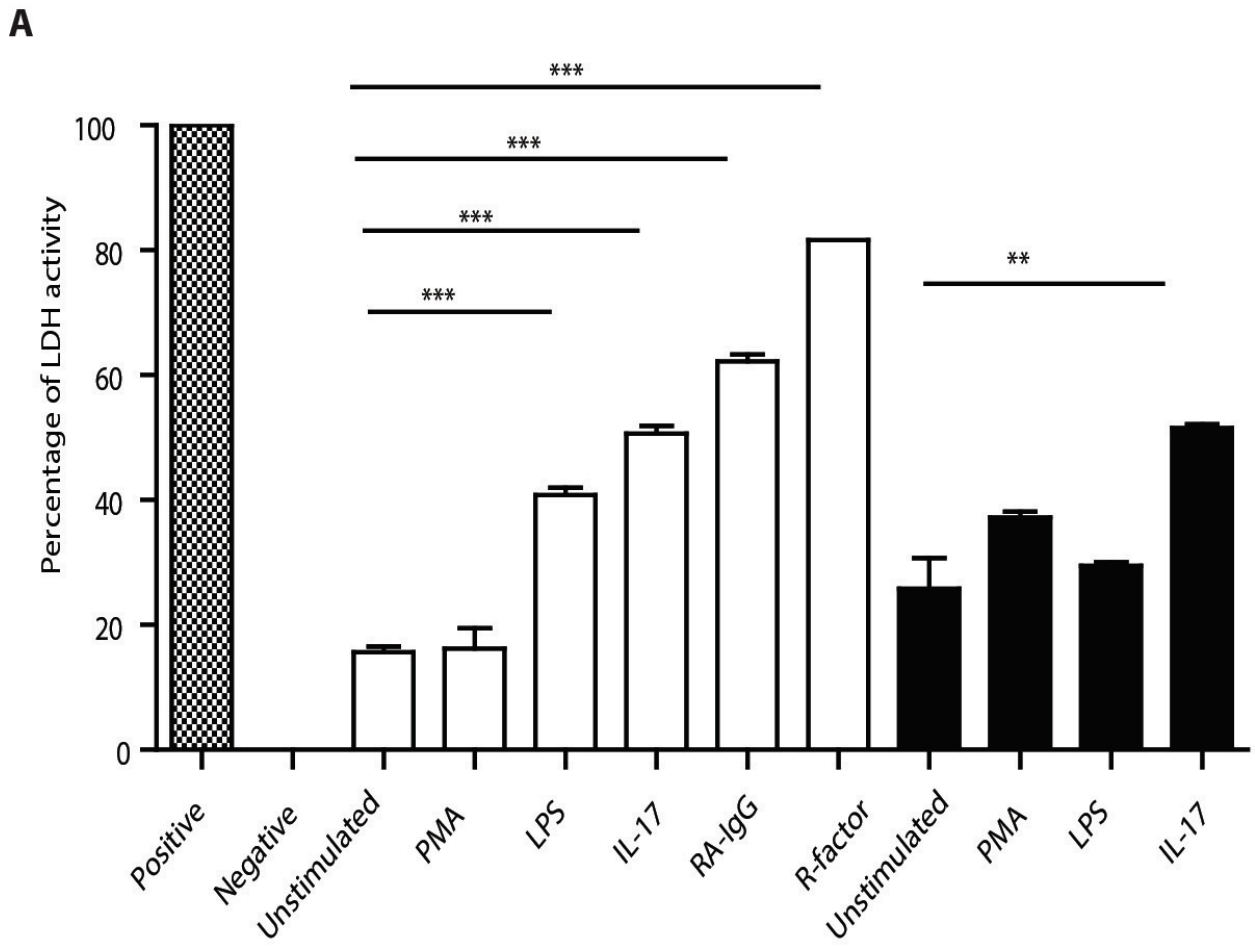


Figure S4. Enhanced NET formation in RA neutrophils and upon exposure to RA-associated cytokines and autoAbs is associated with cell lysis at 1 h in culture. Lactate dehydrogenase (LDH) release was quantified to assess whether NET generation, under conditions described in control and RA neutrophils, is through lytic or non-lytic mechanisms**;** **$p < 0.005$, ***$p < 0.0001$. Results are expressed as percentage mean+SEM of LDH activity in 2 independent experiments performed in triplicate, when comparing to positive control (Triton X).

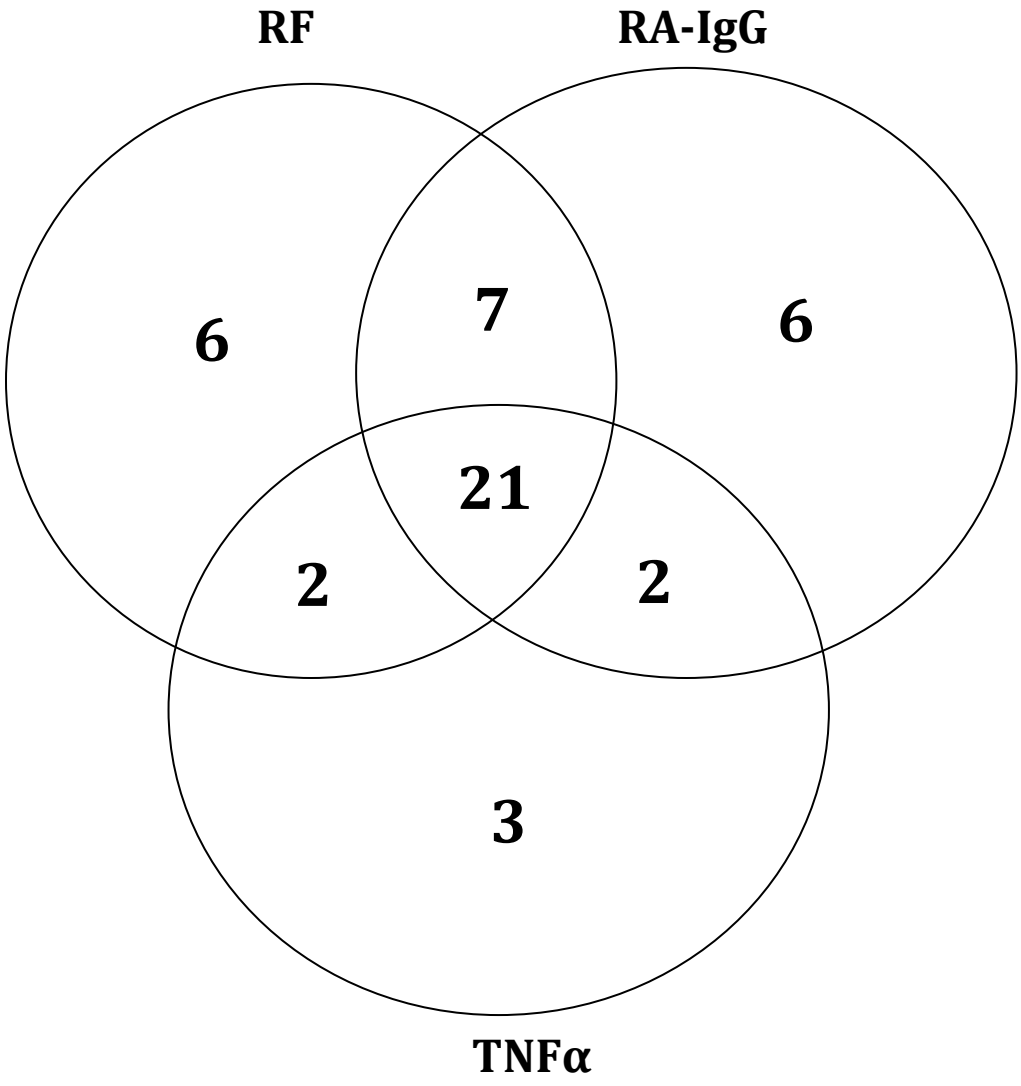


Figure S5. Venn diagram displaying the number of proteins shared or uniquely expressed in the NETs of control neutrophils exposed to rheumatoid factor (RF), IgG isolated from RA serum containing high levels of ACPA (RA IgG) or TNF-α.

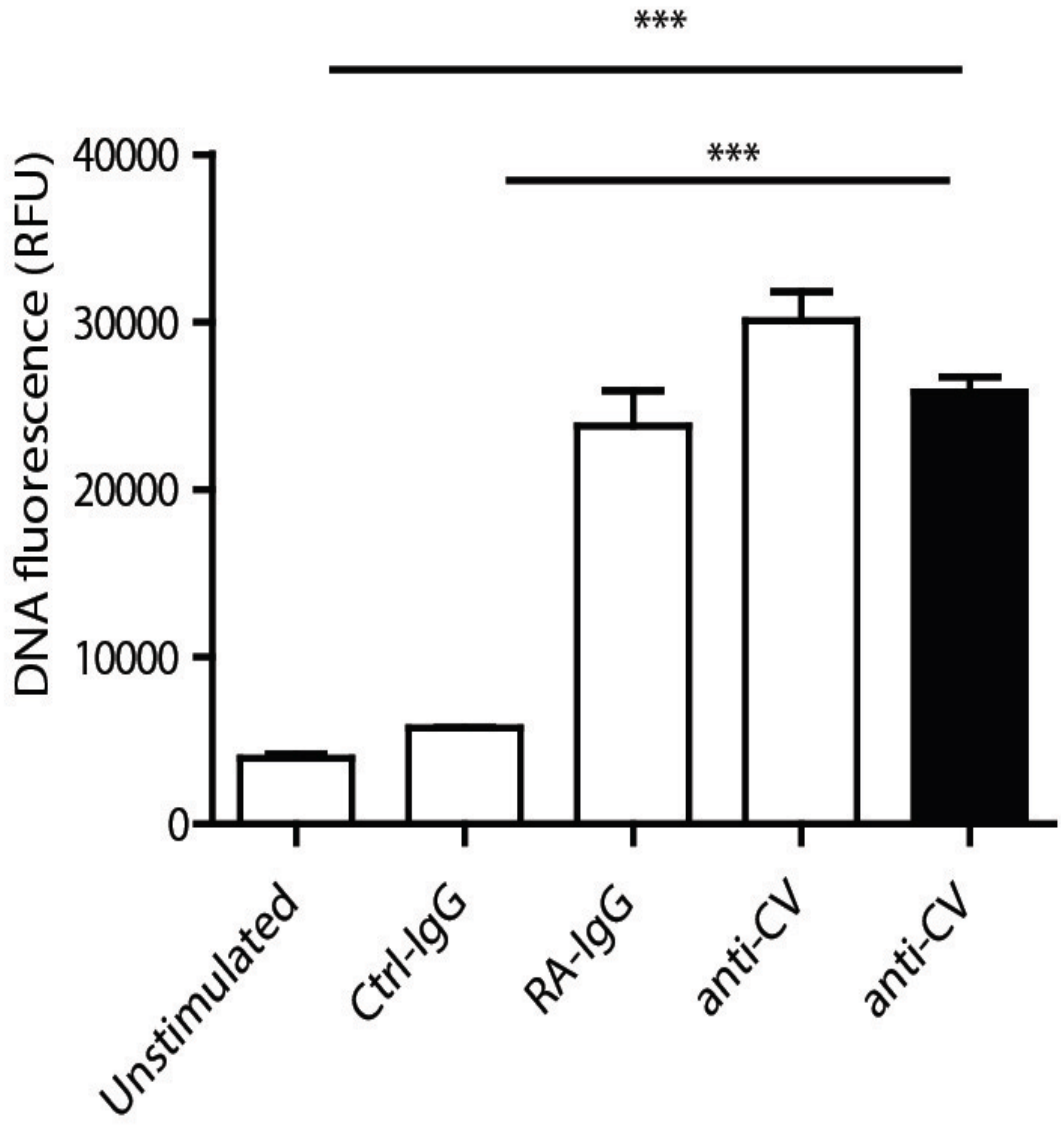


Figure S6. Anti-CV Abs induce NETosis in control (white) and RA (black) neutrophils. Results represent mean+SEM DNA fluorescence quantified by Sytox green assay of 3 experiments, each performed in duplicate-triplicate; ***p<0.001, using two-tailed unpaired t test.

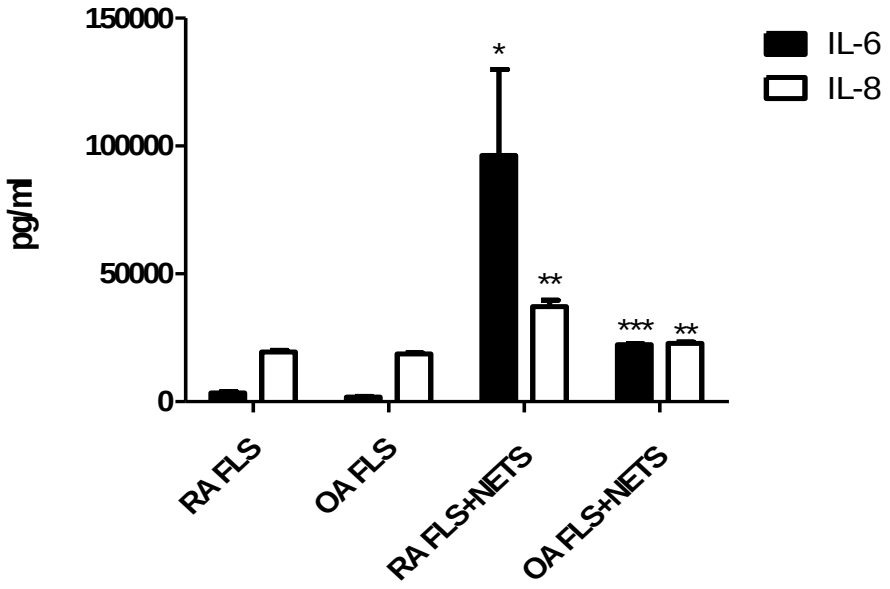


Figure S7. RA NETs induce significant induction of IL-6 and IL-8 secretion at 24 h in RA and OA synovial fibroblasts; Results represent mean+SEM of 2-3 experiments, each performed in triplicate-quadruplicate; ** p<001; *** p<0.001, when compared to FLS without NETs, using two-tailed unpaired t test.

Supplementary Table 1. Demographic and clinical characteristics of patients studied.

Variable	RA (n=55)	Control (n=36)
Age (mean+SD)	52.8±17	49+22
Gender (% females)	73%	79%
Disease duration (years mean+SEM)	11±9	
RF (% positive and mean titer+SEM, IU/mL)	73% (373.5+587 IU/mL)	
ACPA (% positive and mean titer +SEM, U/mL)	75 % (174.8+103 IU/mL)	
ESR (mean+SEM, mm/h)	19.8±20.1	
CRP (mean +SEM, mg/dL)	1.8±2.8	
Medications (% taking)		
Antimalarials	32%	
Prednisone	45%	
Methotrexate	55%	
Sulfasalazine	18%	
Leflunomide	20%	
Anti-TNF biologics	30%	
Rituximab	10%	
Abatacept	4%	

SD: standard deviation; SEM: standard error of mean; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibodies; ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Supplementary Table 2. Association of % PB and/or SF NETosis with clinical and serologic marker of disease activity and with RA medications.

Condition	***r* (p value)**
RA Disease Duration	0.09 (NS)
Joint count	0.07 (NS)
CRP	**0.68 (<0.01)**
ESR	**0.61 (<0.05)**
RF	-0.08 (NS)
ACPA	**0.56 (0.005)**
Serum IL-17	**0.7 (<0.05)**
Serum IL-6	0.28 (NS)
DMARD use	0.12 (NS)
Biologics use	0.08 (NS)
Steroid use	0.07 (NS)

R=correlation coefficient, NS=not significant, CRP=C-reactive protein; WESR= sedimentation rate, ACPA= anticitrullinated peptide Abs, DMARD=disease modifying antirheumatic drugs. Univariate linear regression was performed to determine whether treatment with DMARDS, biologics or steroids was associated with NETosis. Pearson or Spearman's rank correlations were used to examine associations between continuous variables

Supplementary Table 3. Proteins expressed in control NETs upon various sources of stimulation. Proteins that were expressed only upon one specific stimulus were identified in bold characters.

Rheumatoid Factor	**RA IgG**	**TNF**
β-actin	β-Actin	β-Actin
α-actinin-1	**Adenyl-cyclase associated protein**	α-Actinin-1
α-enolase	α-actinin-1	**Actin-related protein 3**
Catalase	α-enolase	α-enolase
Coronin-1	Filamin-A	Filamin-A
Filamin-A	Coronin-1A	Glyceraldehyde-3-phosphate dehydrogenase
Glutathione S-transferase P	Glutathione S-transferase	Glucose-6-phosphate isomerase
Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate dehydrogenase	Histone H2A
Glucose-6-phosphate isomerase	Granulin-7	Histone H2B
Granulin-7	Histone H2A	Histone H4
Histone H2A	Histone H2B	Lactoferrin
Histone H2B	**Histone H3**	Lysozyme
Histone H4	Histone H4	Myeloperoxidase heavy chain
Lactoferrin	Lactoferrin	Myosin 9
Lysozyme C	**Lymphocyte-specific protein-1**	Neutrophil defensin 2
Matrix metalloproteinase-9	**Matrix metalloproteinase-8**	Neutrophil elastase
Moesin	Matrix metalloproteinase-9	Neutrophil gelatinase-associated lipocalin
Myeloperoxidase heavy chain	Myeloperoxidase heavy chain	Plastin-2
Myosin-9	Myosin-9	Profilin-1
Neutrophil defensin 2	Neutrophil defensin 2	Protein S100-A8
Neutrophil elastase	Neutrophil elastase	Protein S100-A9
Neutrophil gelatinase-associated lipocalin	Neutrophil gelatinase-associated lipocalin	Protein S100-P
Olfactomedin-4	Oxidative stress induced growth inhibitor 1	Resistin
Orosomucoid-1	**6-phosphogluconate dehydrogenase, decarboxylating**	Transketolase

Oxidative stress-induced growth inhibitor 1	Peptidoglycan recognition protein 1	YWHAZ
Peptidoglycan recognition protein 1	Plastin-2	**Tropomyosin 3**
Phosphoglycerate mutase	Profilin-1	Vimentin
Plastin-2	Protein S100-A11	**Calmodulin**
Profilin-1	Protein S100-A8	
Protein S100-A11	Protein S100-A9	
Protein S100-A8	Protein S100-P	
Protein S100-A9	Resistin	
Protein S100-P	Transketolase	
Resistin	YWHAZ	
Transaldolase	**Vasodilator-stimulated phosphoprotein**	
Transketolase	Vimentin	